BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT 00/05318

Europäisches Patentamt | Eur pean Patent Office | Office eur péen des brevets

4

10/009436

Bescheinigung | Certificate | Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

99201859.8

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

I.L.C. HATTEN-HECKMAN

DEN HAAG, DEN
THE HAGUE, 10/07/00
LA HAYE, LE

Eur päisches Patentamt
European Patent Office
Office eur péen des brevets

Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

Anmeldung Nr.:
Application no.: 99201859.8
Demande n°:

Anmeldetag:
Date of filing: 10/06/99
Date de dépôt:

Anmelder:
Applicant(s):
Demandeur(s):
Vlaams Interuniversitair Instituut voor Biotechnologie vzw.
9052 Zwijnaarde
BELGIUM

Bezeichnung der Erfindung:
Title of the invention:
Titre de l'invention:
Non-human transgenic animals deficient in gas 6 function and their use

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat: State: Pays:	Tag: Date: Date:	Aktenzeichen: File no. Numéro de dépôt:

Internationale Patentklassifikation:
International Patent classification:
Classification internationale des brevets:
/

Am Anmeldetag benannte Vertragstaaten:
Contracting states designated at date of filing: AT/BE/CH/CY/DE/DK/ES/FI/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE
Etats contractants désignés lors du dépôt:

Bemerkungen:
Remarks:
Remarques:

OAV3 / PCA / GAS6 / 001-044 / EP

Non-human transgenic animals deficient in Gas6 function and th ir use.

The present invention relates to the manufacture of a medicament for the treatment of endothelial dysfunction. The present invention further relates to the use of an inhibitor of the Gas6 function and/or an inhibitor of a Gas6 receptor for the manufacture of said medicament.

The endothelium is uniquely positioned at the interface between the blood and the vessel. As such, it performs multiple functions: it is involved in the regulation of coagulation, leukocyte adhesion in inflammation, vessel tone, and vascular smooth muscle cell growth, and also acts as a barrier to transvascular flux of liquids and solutes wall (Griendling & Alexander, 1996). Far from being a passive participant in these events, it is a dynamic tissue, secreting and modifying vasoactive substances, influencing the behaviour of other cell types, and regulating extracellular matrix production and composition. One of the most important events in the reaction to all forms of injury is adhesion of leukocytes to activated endothelium, a prelude to their emigration into tissues (Cotran & Mayadas-Norton, 1998). Not surprisingly, therefore, when endothelial cells become dysfunctional or deregulated, they may contribute significantly to the development of numerous disorders with major morbidity and mortality, some of which are listed hereunder.

Cardiovascular disorders including atherothrombosis, stroke, arterial (re)-stenosis, ischemic heart disease, myocardial infarction, diabetic macro- and microangiopathy, allograft arteriosclerosis and the like constitute the leading cause of morbidity and mortality in Westernised societies. A common feature of all these disorders is activation of the endothelium, which induces interaction with leukocytes (Ross, 1999). For example, leukocytes adhere to and infiltrate underneath the endothelium during lipid-induced atherosclerosis (Ross, 1999; Libby *et al.*, 1997; Vadas *et al.*,1997; Boyle *et al.*, 1997). During immune-mediated transplant arteriosclerosis or cuff-induced restenosis,

leukocytes adhere to the endothelium, subsequently infiltrate in the intima and media, where they activate medial smooth muscle cells to emigrate in the intima, thereby leading to obstructive intimal thickening (Libby *et al.*,1992). During ischemia/reperfusion, leukocytes have been involved in the production of toxic substances, leading to tissue destruction (Murohara *et al.*, 1995; Dinerman & Mehta, 1990; DeGraba, 1998; Hallenbeck, 1997; Cavanagh *et al.*, 1998).

Excessive formation of new blood vessels (neovascularisation) promotes progression of cancers, induces diabetic retinopathy leading to blindness, and contributes to chronic inflammatory disorders (rheumatoid arthritis, non-healing ulcerations) (Folkman, 1995). In order for endothelial cells to form new blood vessels, they first need to become activated by angiogenic molecules (Carmeliet & Collen, 1998). Leukocytes and tumor cells, adhering to the endothelium, play an essential role in the induction of pathological neovascularization by production of angiogenic factors (Schaper & Ito, 1996). The morbidity and mortality associated with abnormal angiogenesis is significant. Conversely, insufficient angiogenesis has been related to impaired responsiveness of the endothelium, such as in the elderly or diabetic patients.

Infection and resulting sepsis continue to be important causes of morbidity and mortality, in particular in surgical patients. Emerging evidence implicates the endothelium as a central effector in the inflammatory response (Ahmed & Christou, 1996; McGill *et al.*, 1998; Pajkrt & van Deventer, 1996). Through the expression of surface proteins and secretion of soluble mediators, the endothelium controls vascular tone and permeability, regulates coagulation and thrombosis, and directs the passage of leukocytes into areas of inflammation. Derangements in these normal functions may contribute significantly to a maladaptive inflammatory response leading to systemic inflammation, multiple organ failure, and "diffuse intravascular coagulation", a life-threatening complication.

Metastasis involves adhesion of tumor cells to the endothelium with subsequent extravasation and colonisation in distant tissues. Adhesion of

tumor cells is mediated by similar adhesion molecules as used by leukocytes. Endothelial cells in tumors are activated, express more and distinct adhesi n molecules, and are often dysfuntional.

Collectively, a better understanding of the molecular basis leading to activation of endothelial cells is mandated in order to design more rationally future treatments for these life-threatening and debilitating disorders. Considering its enormous surface area (equivalent in adults to an entire soccer field) and accessibility, it is not surprising that this "organ" plays an important role in the above mentioned disorders. The identification of candidates involved in this process may constitute attractive targets for development of drugs for treatment of such disorders. Previous studies using inhibitors or in gene-inactivated mice have amply demonstrated the importance of endothelial activation and endothelial-leukocyte interaction in cardiovascular disorders (Frenette & Wagner, 1997).

Growth arrested gene 6 (Gas6) was originally identified as a gene, whose expression in fibroblasts increased by serum starvation and contact inhibition, and was therefore implicated in reversible growth arrest (Schneider et al., 1988). Gas6 has significant sequence similarity with anticoagulant protein S but lacks anticoagulant activity due to absence of two thrombin cleavage sites. Both molecules are composed of a Gla domain, four EGF-like repeats and a carboxyterminal tandem globular (G)-domains with homology to steroid hormone-binding globulin that are present in molecules involved in cell growth and differentiation (Nakano et al., 1997a).

Gas6 binds members of the Axl family of receptor tyrosine kinases including Axl (also called Ark, Ufo), Sky (also called Rse, Tyro3, Dtk, Etk, Brt) and Mer (also called Eyk, Nyk) (Crosier and Crosier, 1997; Nagata et al., 1996; Stitt et al., 1995). The extracellular domain of these receptors posses Ig-like motifs and fibronectin type III modules, found in cell adhesion molecules. A truncated soluble Axl form is proteolytically generated but it remains unknown whether this soluble receptor acts as a competitive inhibitor for Gas6 by sequestering free ligand, is involved in binding Axl directly and originating a

signal distinct from Gas6, or in inactivating the receptor on the cell surface (Costa et al., 1996).

Gas6 is xpressed in lung and intestin (Manfioletti et al., 1993). Sky is pr dominantly expressed in the brain, while Mer is expressed in monocytes (from peripheral blood and bone marrow) and in cells derived from epithelial tissues (lung, kidney) and reproductive tissues (ovary, prostate), but not in unstimulated granulocytes. Axl is also expressed in monocytes but not in unstimulated granulocytes or mature B or T lymphocytes. Gas6 and Axl are expressed by vascular endothelial cells (Manfioletti et al., 1993).

The function of Gas6 remains largely undetermined and controversial. Gas6 has been reported to inhibit homophilic Axl-mediated aggregation of myeloid cells (Avanzi et al., 1998), but cell-bound Gas6 may mediate aggregation of myeloid cells via interaction with Axl receptor on adjacent cells (McCloskey et al., 1997). Gas6 did not affect adhesion of granulocytes to resting endothelial cells, while it inhibited granulocyte adhesion to TNF-alpha activated endothelial cells, but only at high concentrations (Avanzi et al., 1998). Gas6 is mitogenic for fibroblasts (Goruppi et al., 1996) and Schwann cells (Li et al., 1996) but not for myeloid cells (Avanzi et al., 1997) or endothelial cells (Avanzi et al., 1998). Gas6, induced in injured vascular smooth muscle cells, induces Axl-mediated chemotaxis of smooth muscle cells (Fridell et al., 1998) and, although not mitogenic by itself, enhances the mitogenic activity of thrombin (Fridell et al., 1998). Gas6 also acts as a survival factor for serum-starved fibroblasts (Goruppi et al., 1996) and GnRH neuronal cells (Allen et al., 1999), presumably via activation of PI3-kinase and Akt kinase (Goruppi et al., 1997). Ark signalling protects against apoptosis induced by serum withdrawal or TNF-alpha, but the increased apoptosis of Ark deficient fibroblasts after serum-withdrawal could not be rescued by Gas6 (Bellosta et al., 1997). Gas6 may play a role in clearance of apoptotic cells by forming a bridge between phosphatidylserine on the surface of apoptotic cells and Axl on phagocytotic cells (Nakano et al., 1997b).

Since growth-arrested genes are characteristically expressed when cells undergo growth arrest, the role of Gas6 in endothelial biology was examined. Indeed, endothelial cells become quiescent and enter a stat of prolonged growth arrest (half-life estimated to be ~ 30 years in humans), once vascular development during embryogenesis and postnatal growth is accomplished, and abundantly express Gas6. However, in contrast to other growth-arrested cells (including terminally differentiated cardiomyocytes or neurons), endothelial cells exhibit a unique plasticity to rapidly proliferate and migrate, or to become activated to express prothrombotic factors or leukocyte adhesion molecules. Such responsiveness is essential to accommodate rapidly changing needs to vascularize ischemic tissues, to provide hemostasis upon vascular trauma, to mediate leukocyte extravasation in inflamed tissues or to provide adequate vasomotor control during abnormal tissue perfusion. Notably, the molecular mechanisms that allow quiescent endothelial cells to exhibit such rapid responsiveness remain undetermined.

Collectively, despite the above mentioned data, the precise role of Gas6 and its receptors, in particular in endothelial (patho)-biology *in vivo*, remains largely undetermined.

In this invention it has been surprisingly found that Gas6 deficient endothelial cells are resistant to activation by endotoxin and TNF-α *in vitro* and *in vivo* and that leukocytes did not or much less extravasate in Gas6 deficient mice. This proves that Gas6 plays an essential role in the activation of the endothelium in response to inflammatory stimuli. Since numerous cardiovascular, cancerous and infectious disorders, as indicated above, implicate endothelial activation with subsequent binding and extravasation of leukocytes, Gas6 antagonists and Gas6 receptor inhibitors can be used as compounds for the manufacture of a medicament for the treatment of said disorders.

In a first aspect the present invention concerns an animal deficient in Gas6 function. Preferably, said animal is a transgenic animal in which the Gas6 alleles are inactivated ($Gas6^{-/-}$). More preferably, said animal is a $Gas6^{-/-}$ mouse.

In another aspect the invention relates to the use of said Gas6 deficient animal to study the role of Gas6. Said Gas6 deficient transgenic animals can be used for scre ning compounds having an effect, positive or negative, on said endothelial dysfunction.

In another aspect the invention provides a method to screen for inhibitors of Gas6 function and/or inhibitors of a Gas6 receptor that result in an inhibition of the endothelial activation. Preferably, inhibitors of Gas6 function are soluble forms of the Gas6 receptor and/or Gas6 neutralising antibodies. Alternatively, Gas6 inhibitors may be compounds that prevent the binding of Gas6 to its receptor and/or the activation of said receptor.

In still another aspect, the invention provides the use of an inhibitor of the Gas6 function and/or an inhibitor of a Gas6 receptor for the manufacture of a medicament to treat endothelial dysfunction.

Definitions

The following definitions are set forth to illustrate the meaning and scope of the various terms used to describe the invention therein.

Activation of Gas6 receptor: depends on the type of receptor and is known to the people skilled in the art. For Gas6 tyrosine kinase type receptors, it has been extensively described in WO9628548 and refers to the step of causing the intracellular domain of the receptor to phosphorylate tyrosine residues in a substrate polypeptide.

Inhibitor of Gas6 receptor: any compound that prevent the activation of said receptor.

Gas6 neutralising antibody: any antibody that prevents Gas6 to execute its function.

Inhibitor of Gas6 function: any compound that prevents Gas6 to execute its function. This inhibition may be due to actions such as chemical modification, denaturation of the tertiary structure, complex formation or proteolysis.

Gas6 function: binding of Gas6 with a Gas6 receptor. Bind(ing) means any kind of interaction, be it direct (direct interaction of Gas6 with the

extracellular domain of said receptor) or indirect (interaction of Gas6 with one or more identical and/or non-identical compounds resulting in a complex of which on or more compounds can interact with the extracellular domain) that results in activation of the Gas6 receptor. The extracellular domain means the domain as it occurs in the natural Gas6 receptors, or a fragment thereof, eventually fused to other amino acid sequences, characterised in that it is still able to bind Gas6.

Compound: any chemical or biological compound, including simple inorganic or organic molecules, peptides, peptido-mimetics, proteins, antibodies, carbohydrates, nucleic acids or derivatives thereof.

Examples

Example 1: inhibition of endothelial activation by Gas6 gene-inactivation in mice

In order to study the role of Gas6 *in vivo*, the *Gas6* gene was inactivated in embryonic stem cells via homologous recombination, and Gas6 deficient mice were generated. Surprisingly, these knockout mice developed normally, survived, and were apparently healthy. However, when the Gas6 deficient mice were challenged with inflammatory stimuli, they were found to be protected against endothelial activation.

Eight to twelve week old WT and $Gas6^{-/-}$ mice of either sex with a genetic background of 75% C57Bl6 and 25%129/Sv, and weighing 20-30 g, were used. Animals were maintained in an open animal facility and experiments were performed according to the guidelines of the institutional animal care committee. Adult mice were intraperitoneally injected with endotoxin (0, 10 and 20 mg/kg) and after 12 h, mice were anesthetized by intraperitoneal injection of 60 mg/kg sodium pentobarbital. Blood was collected in citrate via vena cava puncture with a 24-gauge needle. Following centrifugation at 3000 rpm for 10 min, plasma was collected and stored at –20° C until analysis. Factor VII activity was measured via the Coatest FVII assay, as described by the manufacturer (Chromogenix, Brussels, Belgium). The coagulant activity of

the other factors was determined as a clotting time after mixing the murine plasma with human plasma, deficient in the specific factor and addition of thromboplastin (for FII, FV, FVII, FX) or kaolin (FIX) in the University Hospital (UZ Gasthuisberg, Leuven, Belgium). All procoagulant activities were expressed as a percent of the procoagulant activity in a plasma pool of adult wild type mice. Plasma fibrinogen was determined by a coagulation rate assay (Vermylen et al., 1963). For hematological analysis, blood was collected in 0.01 trisodium citrate, and cells were counted using an automated analyzer (Cell-Dyn 610U-Hematology Analyzer, Sequoia-Turner Co, Mountain View, CA). Cell counts are expressed per milliliters of whole blood.

In order to obtain mouse capillary endothelial cells, anesthetized mice were injected s.c. with 500 µl of ice-cold matrigel containing VEGF (100 ng/ml) and heparin (300 µg/ml). After 7 days, the pellet containing neovessels was dissected and enzymatically dispersed using 0.1% type II collagenase (Sigma, St-Louis, Mo). Mouse endothelial cells were routinely cultured in T75 flasks coated with 0.1% gelatin in M131 medium supplemented with 5% MVGS (ref. S00502S; Gibco-BRL). For passage, cells were detached by trypsin/EDTA (0.02-0.05%). For TNF-α stimulation, endothelial cells were refed with fresh culture medium containing 100 ng/ml TNF-α (R&D, Abingdon, UK). After 24 hr, the conditioned medium was harvested and analyzed for the following cytokines: IL-1ß and IL-6 (Quantikine™ M, R&D, Abindon, UK); ELAM, ICAM, VCAM and tissue factor (all from R&D). Recombinant Gas6, produced in 293 monkey kidney cells stably transfected with the pcDNA3 expressing vector encoding the mouse Gas6 cDNA (Grinnell et al., 1990), was supplemented at 200 ng/ml during the experiment as indicated.

For the determination of cytokine concentration after endotoxin injection, adult mice were intraperitoneally injected with endotoxin (20 mg/kg) and after 90 or 180 min, the mice were anesthetized by intraperitoneal injection of 60 mg/kg sodium pentobarbital. Blood was collected in citrate by vena cava puncture with a 24-gauge needle. Following centrifugation at 3000 rpm for 10

min, plasma was collected and stored at –20° C until analysis. Cytokines were measured using Quantikine™ M assays for IL-1ß, IL-6, IL-10 and TNF-α according to the manufacturer's instructions (all from R&D, Abindon, UK).

For apoptosis studies, mouse endothelial cells were cultured in T75 flasks coated with 0.1% gelatin in RPMI 1640 medium containing 10% foetal calf serum (Life Technologies, Paisley, UK), 100 IU penicillin, 100 µg/ml streptomycin, 2 mM glutamine, heparin (100 µg/ml) and endothelial cell growth supplement (30 µg/ml). Apoptosis was induced by (i) supplementation of TNF-α (100 ng/ml; R&D), Fas-ligand (100 ng/ml; R&D), or withdrawal from growth factors (0.1% fetal calf serum). Apoptosis was quantified by measuring cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) using a photometric enzyme immunoassay (Cell Detection ELISA, Boehringer Mannheim, Mannheim, Germany).

Protection against endothelian activation was concluded based on the following criteria:

- Intravenous injection of endotoxin in wild type mice induced strong activation of the coagulation cascade, resulting in reduced levels of factor II, factor V, factor VIII, factor X, and fibrinogen (Table 1). In addition, platelet counts were reduced after endotoxin, indicating formation of platelet-rich thrombi (Table 2). In contrast, endotoxin completely failed to activate coagulation and consume platelets in Gas6 deficient mice, despite comparable increase in plasma levels of IL-6, IL-1ß, IL-10 and TNF-α (Table 3). Nevertheless, Gas6 deficient mice were comparably susceptible to thrombin-induced diffuse intravascular coagulation and death. Upon local injection of endotoxin (50µg) in the footpad, significantly fewer veins developed occluding thrombosis in $Gas6^{-/-}$ than in $Gas6^{+/+}$ mice (Table 4).

When cultured endothelial cells from wild type mice were treated with TNF-α, they expressed increased levels of tissue factor (the initiator of blood coagulation) and plasminogen activator inhibitor-1 (Table 2). In contrast, tissue factor and PAI-1 could not be induced by TNF-α in Gas6 deficient endothelial cells.

- Intravenous injection of endotoxin (10 to 40 mg/kg) in wild type mice reduced circulating numbers of leukocytes, which extravasated in peripheral tissues such as the lung, kidney and heart (Table 2). In contrast, leukocyte numbers in the peripheral blood remained high in Gas6 deficient mice, and leukocytes failed to extravasate. In contrast to cultured wild type endothelial cells, Gas6 deficient endothelial cells failed to express upregulated levels of VCAM, ICAM, and ELAM in response to TNF-α. Further, secretion of IL-1ß and IL-6 was abrogated in Gas6 deficient cells (Table 2). Upon local injection of endotoxin (50 µg) in the footpad significantly fewer leukocytes adhered to the arterial endothelial cells in $Gas6^{-/-}$ than in $Gas6^{+/+}$ mice (Table 4)

- Anti-Fas antibodies induced severe apoptotic liver damage leading to fulminant hepatitits, liver hemorrhage, and death in wild type mice, whereas Gas6 deficient mice were almost completely protected (number of TUNEL positive cells per optical field: 28 ± 7 for $Gas6^{+/+}$ *versus* 8 ± 1 for $Gas6^{-/-}$). Gas6 deficient endothelial cells, unlike wild type cells, are also protected against apoptosis, induced by a cocktail of cytokines (Table 5). Upon local injection of endotoxin (50 µg), in the footpad, significantly fewer apoptotic (TUNEL-positive) endothelial cells were present in $Gas6^{-/-}$ than in $Gas6^{+/+}$ mice (Table 4)

Collectively, these data unveil a novel role for Gas6. Gas6 plays a role in activation of endothelial cells and subsequent binding and extravasation of leukocytes in response to various inflammatory or noxious stimuli. These data illustrate the role of Gas6 in mediating adhesion between activated endothelial and leukocytes.

Example 2: in vivo role of Gas6 in arterial stenosis

A mouse model of arterial stenosis based on the application of perivascular cuff or ligation, is used. Previous studies have indicated that following cuffing or ligation, the endothelium becomes activated, resulting in adhesion of leukocytes to the endothelium, with subsequent infiltration in the intima and media (Kockx *et al.*, 1993). Due to leukocyte production of growth factors and

cytokines, medial smooth muscle cells become activated, proliferate and migrate backwards into the intima, thereby forming a neointima, rich in smooth muscle cells. Two other mouse models of arterial injury, based on mechanical trauma and lectric injury, which result in denudation of the endothelium, are used to study the specific role of Gas6 in activation of the endothelium (Golino *et al.*, 1997).

All models are operational and have been extensively used previously (Carmeliet *etal.*,1997a; Carmeliet *et al.*,1997b; Carmeliet. *et al.*, 1998a; Carmeliet *et al.*, 1997c).

From the results, it is clear that Gas6 deficient mice develop less arterial intimal thickening.

Example 3: in vivo role of Gas6 in atherosclerosis

Cellular interactions between leukocytes and the endothelium are critical events in atherosclerosis and acute coronary syndrome (Ross, 1999). An initial event in atherosclerotic plaque development is the adhesion of leukocytes on activated endothelial surfaces in microregions of disturbed flow (Lüsher *et al.*, 1993; Cosentino & Lüsher, 1998; Dong & Wagner, 1998). When monocytes and endothelial cells are activated via direct cell-cell interaction, both types of cells express adhesion molecules, cytokines, coagulation and fibrinolytic factors, metalloproteinases, and vasoactive substances which contribute to atherogenesis and thrombosis (Ikeda *et al.*, 1998). Since mice do not spontaneously develop atherosclerosis, Gas6 deficient ($Gas6^{-/-}$) mice have been intercrossed with mice deficient in apolipoprotein E ($apoE^{-/-}$), which develop significant atherosclerosis in response to cholesterol feeding. Carmeliet *et al.* (1997d) have previously used the $apoE^{-/-}$ model to study the role of proteinases in atherosclerotic plaque development and aneurysm formation. All the techniques, infrastructure and expertise to study and quantify plaque size, cellular composition, and endothelial activation are known to the people skilled in the

art. The results indicate that Gas6 deficient mice develop smaller atherosclerotic plaques.

Example 4: in vivo role of Gas6 in transplant arteri sclerosis:

Like in atherosclerosis, transplantation of an allograft carotid artery results in the initial activation of the graft endothelium with subsequent adhesion of leukocytes. Moons *et al.* (1999) have previously used this model to study the role of plasminogen in allograft stenosis. All the techniques, infrastructure and expertise to study and quantify intimal thickening due to smooth muscle cell accumulation, cellular composition, and endothelial activation are known to the people skilled in the art. The results indicate that Gas6 deficient mice develop less arteriosclerosis.

Example 5: in vivo role of Gas6 in stroke

A growing body of evidence indicates that inflammatory mechanisms contribute to secondary neuronal injury after acute cerebral ischemia (DeGabra, 1998; Hallenbeck, 1997). Ischemia followed by reperfusion rapidly leads to the expression of inflammatory cytokines particularly TNF-α and IL-1ß, which stimulate a complex cascade of events involving endothelial cells, neurons, astrocytes, and perivascular cells. A secondary response involves activation of the coagulation system and upregulation of cell adhesion molecules. The net effect of these events is transformation of the local endothelium to a prothrombotic/proinflammatory state and induction of leukocyte migration to the site of injury within hours of reperfusion. Leukocytes cause tissue injury by several mechanisms, including occlusion of microvasculature, generation of oxygen free radicals, release of cytotoxic enzymes, alteration of vasomotor reactivity, and increase in cytokine and chemoattractant release. A mouse model of stroke, based on ligation of the middle cerebral artery, was developed to study the role of plasminogen system proteinases *in vivo* (Nagai *et al.*, 1999) and was used to the study the

role of Gas6. Ischemic brain injury was achieved by permanently ligating and sectioning the middle cerebral artery. Infarct size was morphometrically measured after TTc staining of 1 mm thich brain slices within 24 hrs after ligation as described by Nagai *et al.* (1999). Ischemic brain injury was significantly reduced in $Gas6^{-/-}$ as compared to $Gas6^{+/+}$ mice after ligation of the middle cerebral artery (Table 6).

Example 6: role of Gas6 in myocardial ischemia/reperfusion:

The pathogenesis of the reperfusion syndrome is complex and as yet not fully elucidated. During reperfusion of ischemic myocardium, there is a well-orchestrated interplay between the coronary vascular endothelium and the circulating neutrophils (Murohara *et al.*,1995; Dinerman & Mehta, 1990). This interplay involves initial "rolling" of neutrophils along the endothelium during the early moments of reperfusion, followed by firm attachment and diapedesis into the myocardial parenchyma where neutrophil-myocyte interaction contributes to the necrotic process (Murohara *et al.*, 1995). Reperfusion injury is characterized by increased microvascular permeability, oedema and tissue necrosis, and is associated with free radical release, cellular calcium overload and activation of neutrophils (Cavanagh, 1998). The results indicate that Gas6 deficient mice develop less significant ischemic myocardial reperfusion injury.

Example 7: in vivo role of Gas6 in angiogenesis

Unlike their active growth and remodeling during embryonic development, endothelial cells in the adult are quiescent, and need to become activated when new blood vessels are formed. Over the last decade, remarkable progress has been made in the identification of a variety of molecules that stimulate or inhibit angiogenesis *in vivo*. Angiogenesis inhibitors have attained significant interest for the treatment of cancer, inflammatory disorders and diabetic retinopathy, as endothelial cells that invade tumors are

non-tumoral host-derived cells, that would not become resistant to chemotherapy (Folkman, 1998). The significant suppression of angiogenesis in Gas6 defici nt mice suggests that Gas6, somehow, is required for endothelial activation during angiogenesis, and that Gas6 antagonists may be useful angiogenesis inhibitors. The role of Gas6 in angiogenesis was further studied using other models of angiogenesis: (i) a tumor angiogenesis model whereby invasion of tumor cells on implanted collagen cells in dorsal skin chambers is dependent on angiogenic ingrowth of capillaries in the tumor, as used previously by us for the study of the role of PAI-1 (Bajou *et al,* 1998); (ii) neovascularization after retinal ischemia (operational); (iii) angiogenesis in embryonic stem cell-derived tumors, that are wild type or deficient for Gas6, as used to study the role of HIF(Carmeliet *et al,* 1998b); (iv) neovascularization after occlusion of the femoral artery (Couffinhal *et al.,* 1998) or (v) of the coronary artery.

Ingrowth of capillaries in matrigel was performed as described by Passanti *et al.* (1992): Briefly, 500 µl ice-cold matrigel containing heparin (300 µg/ml) and rVEGF$_{165}$ (100ng/ml) was injected subcutaneaously into the groin. After 7 days, the matrigel pellet with the neovessels was dissected for analysis of neovascularisation: one part was homogenized to determine the hemoglobin content determined using Drabkin's reagent (Sigma St. Quentin Fallavier, France), whereas the other part was fixed in 1% paraformaldehyde for histological analysis. Subcutaneous injection of matrigel containing VEGF induced a strong angiogenic ingrowth of capillaries in wild type mice, while this angiogenic response was inhibited for more than 80% in Gas6 deficient mice (Table 7).

Depth of tumoral sprouts was measured by plating malignant murine keratinocytes (PDVA cells) on collagen gel (4 mg/ml of type I collagen isolated from rat tail tendons) inserted in Teflon rings. Cell coated collagen gels were then recovered with a silicone transplantation chamber and implanted in toto onto the dorsal muscle fascia of mice, as described by Bajou *et al.* (1998). Tumor bearing mice were killed after 21 days, tumors were dissected and processed for histology. The maximum depth of

individula tumor sprouts, known to depend on concommitant ingrowth of host-derived capillaries in the invading tumor (Bajou *et al.* 1998) was measured by image analysis. For measurement of the limb-ischemia induced formation of collaterals, the right femoral artery was occluded distal to the branch site of the deep femoral and of the popliteal artery to guarantee complete occlusion. To document the recovery of the pelvic limb blood flow, in vivo infra-red thermography (IRT, Varioscan 3011, Jenoptik, Germany) was used before, immediately after and within one, two, three and seven days after femoral occlusion. The mice were anesthetized, kept five minutes on a heating pad at 37°C and then mounted on a cork plate and studied with IRT at ambient temperature of 20°C. Temperature changes were evaluated at standardized locations on the left and right feet using IRBIS Plus software. The temperature difference between occluded and non-occluded limbs was calculated at standardized temperatures of the non-occluded foot (at 24, 25, 26, 27, 28, 29 and 30 °C) before, immediately after and at the indicated days after ligation.

Tumor angiogenesis and formation of collaterals after limb ischemia were significantly reduced in $Gas6^{-/-}$ as compared to $Gas6^{+/+}$ mice (Table 8, Table 9).

Example 8: inhibition of endothelial activation by soluble Axl receptor

Mice with deficiency of Gas6 are used to test the potential of Gas6 antagonists for the treatment of the above mentioned models of cardiovascular disorders and angiogenesis. To further evaluate the use of Gas6 inhibitors in therapy, a recombinant adenovirus is generated that expresses a soluble Ark (the mouse Gas6 receptor) (Costa *et al.*, 1996), that has been demonstrated to block the action of Gas6 *in vitro* and *in vivo*. The technniques for recombinant adenoviruses in mice are described by Carmeliet *et al.* (1997b, 1997e). The use of recombinant adenoviruses that result in the expression of the transferred genes for at least 10 days shows that this inhibitor has a positive (therapeutic) effect during arterial stenosis, allograft reaction, stroke, myocardial ischemia/reperfusion, and angiogenesis.

Example 9: chemical Gas6 inhibitors

Novel chemical Gas6 inhibitors ar screened by high throughput screening as described by Fernandes (1998) and Kenny et al. (1998).

References

Ahmed, N. & Christou, N. Systemic inflammatory response syndrome: interactions between immune cells and the endothelium. *Shock* **6**, S39-42 (1996).

Allen, M.P. *et al.* Growth arrest-specific gene 6 (Gas6)/adhesion related kinase (Ark) signaling promotes gonadotropin-releasing hormone neuronal survival via extracellular signal-regulated kinase (ERK) and Akt [In Process Citation]. *Mol Endocrinol* **13**, 191-201 (1999).

Avanzi, G.C. *et al.* GAS6, the ligand of Axl and Rse receptors, is expressed in hematopoietic tissue but lacks mitogenic activity. *Exp Hematol* **25**, 1219-1226 (1997).

Avanzi, G.C. *et al.* GAS6 inhibits granulocyte adhesion to endothelial cells. *Blood* **91**, 2334-2340 (1998).

Bajou, K. *et al.* Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. *Nat Med* **4**, 923-928 (1998).

Bellosta, P., Costa, M., Lin, D.A. & Basilico, C. The receptor tyrosine kinase ARK mediates cell aggregation by homophilic binding. *Mol Cell Biol* **15**, 614-625 (1995).

Bellosta, P., Zhang, Q., Goff, S.P. & Basilico, C. Signaling through the ARK tyrosine kinase receptor protects from apoptosis in the absence of growth stimulation. *Oncogene* **15**, 2387-2397 (1997).

Boyle, E.M., Jr., Lille, S.T., Allaire, E., Clowes, A.W. & Verrier, E.D. Endothelial cell injury in cardiovascular surgery: atherosclerosis. *Ann Thorac Surg* **63**, 885-894 (1997).

Carmeliet, P., Moons, L., Ploplis, V., Plow, E. & Collen, D. Impaired arterial neointima formation in mice with disruption of the plasminogen gene. *J Clin Invest* **99**, 200-208 (1997a).

Carmeliet, P. *et al.* Inhibitory role of plasminogen activator inhibitor-1 in arterial wound healing and neointima formation: a gene targeting and gene transfer study in mice [see comments]. *Circulation* **96**, 3180-3191 (1997b).

Carmeliet, P. *et al.* Urokinase-type but not tissue-type plasminogen activator mediates arterial neointima formation in mice. *Circ Res* **81**, 829-839 (1997c).

Carmeliet, P. *et al.* Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. *Nat Genet* **17**, 439-444 (1997d).

Carmeliet, P., Stassen, J.M., Meidell, R., Collen, D. & Gerard, R. Adenovirus-mediated gene transfer of rt-PA restores thrombolysis in t-PA deficient mice. *Blood* **90**, 1527-1534 (1997e).

Carmeliet, P. & Collen, D. Vascular development and disorders: molecular analysis and pathogenetic insights. *Kidney Int* **53**, 1519-1549 (1998).

Carmeliet, P. *et al.* Receptor-independent role of urokinase-type plasminogen activator in arterial wound healing and intima formation in mice. *J Cell Biol* **140**, 233-245 (1998a).

Carmeliet, P. *et al.* Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation, and tumor angiogenesis. *Nature* **394**, 485-490 (1998b).

Cavanagh, S.P., Gough, M.J. & Homer-Vanniasinkam, S. The role of the neutrophil in ischaemia-reperfusion injury: potential therapeutic interventions. *Cardiovasc Surg* **6**, 112-118 (1998).

Cosentino, F. & Luscher, T.F. Endothelial dysfunction in diabetes mellitus [In Process Citation]. *J Cardiovasc Pharmacol* **32**, S54-61 (1998).

Costa, M., Bellosta, P. & Basilico, C. Cleavage and release of a soluble form of the receptor tyrosine kinase ARK in vitro and in vivo. *J Cell Physiol* **168**, 737-744 (1996).

Cotran, R.S. & Mayadas-Norton, T. Endothelial adhesion molecules in health and disease. *Pathol Biol (Paris)* **46**, 164-170 (1998).

Couffinhal, T. *et al.* Mouse model of angiogenesis. *Am J Pathol* **152**, 1667-1679 (1998).

Crosier, K.E. & Crosier, P.S. New insights into the control of cell growth; the role of the Axl family. *Pathology* **29**, 131-135 (1997).

DeGraba, T.J. The role of inflammation after acute stroke: utility of pursuing anti- adhesion molecule therapy. *Neurology* **51**, S62-68 (1998).

Dinerman, J.L. & Mehta, J.L. Endothelial, platelet and leukocyte interactions in ischemic heart disease: insights into potential mechanisms and their clinical relevance [see comments]. *J Am Coll Cardiol* **16**, 207-222 (1990).

Dong, Z.M. & Wagner, D.D. Leukocyte-endothelium adhesion molecules in atherosclerosis. *J Lab Clin Med* **132**, 369-375 (1998).

Fernandes, P. B. (1998). Technological advances in high-throughput screening. Curr Opin Chem Biol *2*, 597-603

Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nature Med* **1**, 27-31 (1995).

Folkman, J. Antiangiogenic gene therapy. *Proc Natl Acad Sci U S A* **95**, 9064-9066 (1998).

Fridell, Y.W., Villa, J., Jr., Attar, E.C. & Liu, E.T. GAS6 induces Axl-mediated chemotaxis of vascular smooth muscle cells. *J Biol Chem* **273**, 7123-7126 (1998).

Frenette, P.S. & Wagner, D.D. Insights into selectin function from knockout mice. *Thromb Haemost* **78**, 60-64 (1997).

Golino, P. *et al.* Inhibition of leucocyte and platelet adhesion reduces neointimal hyperplasia after arterial injury. *Thromb Haemost* **77**, 783-788 (1997).

Goruppi, S., Ruaro, E. & Schneider, C. Gas6, the ligand of Axl tyrosine kinase receptor, has mitogenic and survival activities for serum starved NIH3T3 fibroblasts. *Oncogene* **12**, 471-480 (1996).

Goruppi, S., Ruaro, E., Varnum, B. & Schneider, C. Requirement of phosphatidylinositol 3-kinase-dependent pathway and Src for Gas6-Axl mitogenic and survival activities in NIH 3T3 fibroblasts. *Mol Cell Biol* **17**, 4442-4453 (1997).

Griendling, K.K. & Alexander, R.W. Endothelial control of the cardiovascular syst m: recent advances. *Faseb J* **10**, 283-292 (1996).

Hallenbeck, J.M. Cytokines, macrophag s, and leukocytes in brain ischemia. *Neurology* **49**, S5-9 (1997).

Ikeda, U., Takahashi, M. & Shimada, K. Monocyte-endothelial cell interaction in ath rogenesis and thrombosis. *Clin Cardiol* **21**, 11-14 (1998).

Kenny, B. A., Bushfield, M., Parry-Smith, D. J., Fogarty, S., and Treherne, J. M. (1998). The application of high-throughput screening to novel lead discovery. Prog Drug Res *51*, 245-69.

Kockx, M.M. *et al.* The endothelium during cuff-induced neointima formation in the rabbit carotid artery. *Arterioscler Thromb* **13**, 1874-1884 (1993).

Li, R. *et al.* Identification of Gas6 as a growth factor for human Schwann cells. *J Neurosci* **16**, 2012-2019 (1996).

Libby, P., Schwartz, D., Brogi, E., Tanaka, H. & Clinton, S.K. A cascade model for restenosis. A special case of atherosclerosis progression. *Circulation* **86**, III47-52 (1992).

Libby, P., Sukhova, G., Lee, R.T. & Liao, J.K. Molecular biology of atherosclerosis. *Int J Cardiol* **62 Suppl 2**, S23-29 (1997).

Lüsher, T.F., Tanner, F.C., Tschudi, M.R. & Noll, G. Endothelial dysfunction in coronary artery disease. *Annu Rev Med* **44**, 395-418 (1993).

Manfioletti, G., Brancolini, C., Avanzi, G. & Schneider, C. The protein encoded by a growth arrest-specific gene (gas6) is a new member of the vitamin K-dependent proteins related to protein S, a negative coregulator in the blood-coagulation cascade. *Mol Cell Biol* **13**, 4976-4985 (1993).

McCloskey, P. *et al.* GAS6 mediates adhesion of cells expressing the receptor tyrosine kinase Axl. *J Biol Chem* **272**, 23285-23291 (1997).

McGill, S.N., Ahmed, N.A. & Christou, N.V. Endothelial cells: role in infection and inflammation. *World J Surg* **22**, 171-178 (1998).

Moons, L. *et al.* Reduced transplant arteriosclerosis in plasminogen deficient mice. *J Clin Invest* (in press) (1998).

Murohara, T., Guo, J.P. & Lefer, A.M. Cardioprotection by a novel recombinant serine protease inhibitor in myocardial ischemia and reperfusion injury. *J Pharmacol Exp Ther* **274**, 1246-1253 (1995).

Nagai, N., De Mol, M., Lijnen, H.R., Carmeliet, P. & Collen, D. Role of plasminogen system components in focal cerebral ischemic infarction. A gene targeting and gene transfer study in mice. *Circulation* (in press) (1999).

Nagata, K. *et al.* Identification of the product of growth arrest-specific gene 6 as a common ligand for Axl, Sky, and Mer receptor tyrosine kinases. *J Biol Chem* **271**, 30022-30027 (1996).

Nakano, T. *et al.* Requirement of gamma-carboxyglutamic acid residues for the biological activity of Gas6: contribution of endogenous Gas6 to the proliferation of vascular smooth muscle cells. *Biochem J* **323**, 387-392 (1997a).

Nakano, T. *et al.* Cell adhesion to phosphatidylserine mediated by a product of growth arrest-specific gene 6. *J Biol Chem* **272**, 29411-29414 (1997b).

Pajkrt, D. & van Deventer, S.J. The cellular response in sepsis. *Curr Top Microbiol Immunol* **216**, 119-132 (1996).

Ross, R. Atherosclerosis–an inflammatory disease. *N Engl J Med* **340**, 115-126 (1999).

Schaper, W. & Ito, W.D. Molecular mechanisms of coronary collateral vessel growth. *Circ Res* **79**, 911-919 (1996).

Schneider, C., King, R.M. & Philipson, L. Genes specifically expressed at growth arrest of mammalian cells. *Cell* **54**, 787-793 (1988).

Stitt, T.N. *et al.* The anticoagulation factor protein S and its relative, Gas6, are ligands for the Tyro 3/Axl family of receptor tyrosine kinases. *Cell* **80**, 661-670 (1995).

Vadas, M.A., Gamble, J.R., Rye, K. & Barter, P. Regulation of leucocyte-endothelial interactions of special relevance to atherogenesis. *Clin Exp Pharmacol Physiol* **24**, A33-35 (1997)..

Table 1: Coagulation and hematological parameters after endotoxin injection (20 mg/kg).

	Dos LPS	$Gas6^{+/+}$	$Gas6^{-/-}$
Factor II	0	102 ± 13	105 ± 8
	10	76 ± 7	100 ± 10
	20	45 ± 6.3	94 ± 1
Factor V	0	89 ± 20	84 ± 8
	10	42 ± 4	84 ± 9
	20	29 ± 6	84 ± 6
Factor VIII	0	71 ± 15	78 ± 17
	10	30 ± 9	94 ± 16
	20	30 ± 4	118 ± 11
Factor X	0	97 ± 10	87 ± 5
	10	67 ± 4	87 ± 8
	20	44 ± 4	92 ± 4
Fibrinogen	0	122 ± 40	88 ± 13
	10	83 ± 14	170 ± 13
	20	66 ± 21	211 ± 2
Platelets	0	390 ± 50	515 ± 60
	10	370 ± 50	455 ± 50
	20	250 ± 40	547 ± 30
Leukocytes	0	2.6 ± 0.3	3.4 ± 0.6
	10	2.6 ± 0.7	4.0 ± 0.3
	20	1.5 ± 0.3	4.3 ± 1.3

Data represent mean ± SD (5 mice) of plasma concentrations of coagulation factors (expressed in % of normal pool), platelets and leukocytes in mice within 12 h after injection of endotoxin (0, 10 and 20 mg/kg).

Tabl 2: Activation of endothelial cells in vitro.

	Dose TNF-α	Gas6$^{+/+}$	Gas6$^{-/-}$ saline	Gas6$^{-/-}$ + Gas6 (200 ng/ml)
ELAM	**0** **100**	1 ± 0.3 11 ± 2	1 ± 0.4 1 ± 16	1 ± 0.3 10 ± 1
ICAM	**0** **100**	0.4 ± 0.02 8 ± 1	0.5 ± 0.01 0.8 ± 0.11	0.3 ± 0.1 7.5 ± 1
VCAM	**0** **100**	2 ± 0.2 14 ± 2	2 ± 1 3 ± 0.4	2 ± 1 9 ± 2
IL-6	**0** **100**	0.6 ± 0.1 6 ± 1	0.5 ± 0.03 0.9 ± 0.1	N.D. N.D.
IL-1ß	**0** **100**	1 ± 0.1 11 ± 1	N.D. 2 ± 1	N.D. N.D.
Tissue Factor	**0** **100**	N.D. 8 ± 1	N.D. 1 ± 0.3	N.D. 8 ± 2

Data represent mean ± SD (9) of cytokine concentrations (ng/10^6 cells).

Table 3: Serum cytokine concentration after endotoxin injection (20 mg/kg).

	Tim (min)	$Gas6^{+/+}$	$Gas6^{-/-}$
TNF-α (ng/ml)	90	9 ± 4	13 ± 6
	180	1.4 ± 0.4	1.3 ± 1
IL-6 (ng/ml)	90	22 ± 14	28 ± 12
	180	83 ± 24	105 ± 25
IL-1ß (pg/ml)	90	66 ± 2	136 ± 100
	180	146 ± 138	430 ± 200
IL-10 (pg/ml)	90	1260 ± 590	980 ± 350
	180	560 ± 130	530 ± 150

Data represent mean ± SD (7 mice) of cytokine serum concentrations in mice after injection of endotoxin (20 mg/kg).

Table 4: Endotoxin injection in the footpad

All values in $Gas6^{-/-}$ mice are significantly different from those in $Gas6^{+/+}$ mice ($p \leq 0.05$).

	$Gas6^{+/+}$	$Gas6^{-/-}$
% Veins without thrombosis	15 ± 3	60 ± 6
% Veins with thrombosis	85 ± 3	40 ± 6
% Arteries without leukocytes	61 ± 10	100 ± 0
% Arteries with leukocytes	39 ± 10	0 ± 0
% TUNEL-positive endothelial cells	4.5 ± 0.6	1.2 ± 0.3

Table 5: Apoptosis of cultured endothelial cells

	Ga $6^{+/+}$	$Gas6^{-/-}$
Control	23 ± 4	23 ± 4
TNF-α (100 ng/ml)	220 ± 15	54 ± 5
Growth factor deprivation	200 ± 11	49 ± 4
Fas-ligand (100 ng/ml)	27 ± 4	23 ± 2

Data represent mean ± SD (n=9) of oligonucleosomes/10^5 cells.

Table 6: Ischemic brain injury (stroke)

	$Gas6^{+/+}$	$Gas6^{-/-}$
Infarct size (mm3)	19 ± 2	10 ± 2

All values in $Gas6^{-/-}$ mice are significantly different from those in $Gas6^{+/+}$ mice ($p<0.05$)

Table 7: Matrigel Angiogenesis

	$Gas6^{+/+}$	$Gas6^{-/-}$
Hemoglobin content (g/dl)	0.28 ± 0.02	0.02 ± 0.02*

*: $p<0.05$ versus $Gas6^{+/+}$.

Table 8: Tumoral angiogenesis

	$Gas6^{+/+}$	$Gas6^{-/-}$
Depth of tumoral sprouts (µm)	425 ± 30	130 ± 30*

*: $p<0.05$ versus $Gas6^{+/+}$.

Table 9: Limb-ischemia induced formation of collaterals.

Time of analysis	$Gas6^{+/+}$	$Gas6^{-/-}$
Before occlusion	0.5 ± 0.2	0.7 ± 0.4
Immediately after occlusion	2.4 ± 0.3	2.4 ± 0.4
1 day after occlusion	1.2 ± 0.3	1.7 ± 0.1*
2 day after occlusion	1.2 ± 0.2	2.0 ± 0.2*
3 day after occlusion	0.7 ± 0.2	1.5 ± 0.2*
7 day after occlusion	0.5 ± 0.2	1.5 ± 0.4*

Data represent mean ± SD (n=9) of the difference in temperature between occluded and non-occluded limbs. *: $p<0.05$ versus $Gas6^{+/+}$.

Claims

1. Non-human transgenic animal deficient in Gas6 function.

2. Non-human transg nic animal according to claim 1 in which said animal is Gas6$^{-/-}$.

3. Non-human transgenic animal according to claim 1 or 2 in which said animal is a mouse.

4. Use of an animal according to claim 1-3 to study the role of Gas6 in endothelial activation.

5. Use of an inhibitor of the Gas6 function and/or an inhibitor of a Gas6 receptor for the manufacture of a medicament for the treatment of endothelial dysfunction.

6. Use according to claim 5 in which endothelial dysfunction results in a cardiovascular disorder such as atherothrombosis, stroke, arterial stenosis, arterial restenosis, ischemic heart disease, mycardial infarction, diabetic macroangiopathy, diabetic microangiopathy and/or allograft arteriosclerosis.

7. Use according to claim 5 in which endothelial dysfunction results in abnormal angiogenesis such as diabetic retinopathy.

8. Use according to claim 5-7 in which the Gas6 receptor is AXL, MER and/or RSE.

9. Use according to claim 8 in which the inhibitor is a soluble form of the AXL, MER or RSE receptor.

10. Use according to claim 5-8 in which the inhibitor is a Gas6 neutralising antibody.

Abstract

The pres nt invention relates to the manufacture of a medicament for the treatment of endothelial dysfunction. The present invention further relates to the us of an inhibitor of the Gas6 function and/or an inhibitor of a Gas6 receptor for the manufacture of said medicament.

It has been shown that Gas6 deficient endothelial cells are resistant to activation by endotoxin and TNF-α *in vitro* and *in vivo* and that leukocytes did not or much less extravasate in Gas6 deficient mice.